ApoE2-associated hypertriglyceridemia is ameliorated by increased levels of apoA-V but unaffected by apoC-III deficiency

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Abstract **Apolipoprotein E2 (apoE2)-associated hyperlipidemia is characterized by a disturbed clearance of apoE2-enriched VLDL remnants. Because excess apoE2 inhibits LPL-mediated triglyceride (TG) hydrolysis in vitro, we investigated whether direct or indirect stimulation of LPL activity in vivo reduces the apoE2-associated hypertriglyceridemia. Here, we studied the role of LPL and two potent modifiers, the LPL inhibitor apoC-III and the LPL activator apoA-V, in *APOE2*-knockin (*APOE2*) mice. Injection of heparin in *APOE2* mice reduced plasma TG by 53% and plasma total cholesterol (TC) by 18%. Adenovirus-mediated overexpression of LPL reduced plasma TG by 85% and TC by 40%. Both experiments indicate that the TG in apoE2-enriched particles is a suitable substrate for LPL. Indirect activation of LPL activity via deletion of *Apoc3* in *APOE2* mice did not affect plasma TG levels, whereas overexpression of *Apoa5* in *APOE2* mice did reduce plasma TG by 81% and plasma TC by 41%. In conclusion, the hypertriglyceridemia in *APOE2* mice can be ameliorated by the direct activation of LPL activity. Indirect activation of LPL via overexpression of apoA-V does, whereas deletion of apoC-III does not, affect the plasma TGs in *APOE2* mice. These data indicate that changes in apoA-V levels have a dominant effect over changes in apoC-III levels in the improvement of *APOE2*-associated hypertriglyceridemia.**—Gerritsen, G., C. C. van der Hoogt, F. G. Schaap, P. J. Voshol, K. E. Kypreos, N. Maeda, A. K. Groen, L. M. Havekes, P. C. N. Rensen, and K. W. van Dijk. **ApoE2-associated hypertriglyceridemia is ameliorated by increased levels of apoA-V but unaffected by apoC-III deficiency.** ***J. Lipid Res.*** **2008.** 49: **1048–1055.**

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Apolipoprotein E2 (apoE2)-associated hyperlipidemia is characterized by increased plasma levels of chylomicron and VLDL remnants and is associated with xanthomatosis and premature atherosclerosis (1). ApoE2 has a single amino acid substitution (arginine158→cysteine) compared with the common apoE3 variant, resulting in a low binding affinity for the LDL receptor (2, 3). In vivo, this is associated with impaired hepatic clearance of VLDL and chylomicron remnant particles (4), resulting in increased plasma triglyceride (TG) and total cholesterol (TC) levels. Simultaneously, apoE2 accumulates in plasma, leading to an increase in apoE-mediated inhibition of LPL-mediated TG hydrolysis (5). It has been postulated that both impaired remnant clearance and impaired remnant generation via lipolysis contribute to the hyperlipidemia associated with apoE2 (5).

We and others have found that VLDL obtained from hyperlipidemic patients homozygous for *APOE2* is a relatively poor substrate for LPL-mediated lipolysis (6). Two potent modifiers of LPL activity have been described,

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Abbreviations: apoE2, apolipoprotein E2; FPLC, fast-protein liquid chromatography; HSPG, heparan sulfate proteoglycan; TC, total cholesterol; TG, triglyceride.

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apoA-V and apoC-III, that are encoded in the same gene cluster on chromosome 11 (7). In vitro and in vivo mouse studies indicate that apoA-V stimulates LPL-mediated TG hydrolysis and that apoC-III inhibits this process (8–12). Overexpression of apoA-V in mice reduces plasma TG levels via the stimulation of LPL activity (13), and overexpression of apoC-III results in increased plasma TG levels via the inhibition of LPL (14). Studies in *Apoc3*-knockout mice show accelerated LPL-mediated TG hydrolysis (15, 16). Deficiency in apoA-V in both mice and humans is associated with hypertriglyceridemia (17–19).

In the present study, we investigated the role of LPL-mediated TG hydrolysis in apoE2-associated hyperlipidemia in vivo. A direct increase of active LPL in the circulation of *APOE2*-knockin (*APOE2*) mice via heparin injection and via adenovirus-mediated gene transfer of LPL reduced both TG and TC levels. Indirect stimulation of the LPL activity via the deletion of endogenous *Apoc3* did not affect the plasma TG levels, whereas indirect stimulation via adenovirus-mediated overexpression of *Apoa5* resulted in decreased plasma TG and TC levels. Thus, stimulation of LPL activity via apoA-V overexpression or apoC-III deficiency occurs via different mechanisms.

METHODS

Adenoviral constructs

The adenoviral vectors expressing enzymatically active LPL (Ad*LPL*) and inactive LPL (Ad*LPL*-inactive) were kindly provided by Dr. S. Santamarina-Fojo (20). The generation of the adenoviral vectors expressing apoA-V (Ad*Apoa5*), the control empty vector (AdEmpty), and β-galactosidase (AdlacZ) has been described (8, 13). Expansion, purification, and titration of the adenoviral vectors were performed as described previously (21). Before in vivo administration, the adenoviral vectors were diluted to a dose of 5×10^8 plaque-forming units (pfu) in 200 μl of sterile PBS.

Mouse models

APOE2-knockin mice, carrying the human *APOE2* gene in place of the mouse *Apoe* gene, have been described (22). These mice were backcrossed eight times with C57BL/6 mice to achieve a more homogenous genetic background and subsequently intercrossed to obtain homozygous *APOE2* mice. *Apoc3*$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and intercrossed with *APOE2* mice to obtain *APOE2*, *APOE2.Apoc3*$^{+/-}$, and *APOE2.Apoc3*$^{-/-}$ mice. The mice were fed a regular mouse diet (SRM-A; Hope Farms, Woerden, The Netherlands) and given free access to food and water. At least 5 days before adenovirus injection, mice were transferred to filter-top cages in designated rooms. All animal experimentation protocols were approved by the Committee on Animal Experimentation of the Leiden University Medical Center.

Adenovirus-mediated gene transfer in mice

Male *APOE2* mice at 13–18 weeks of age were selected for injection with Ad*LPL* or Ad*LPL*-inactive. A dose of 5×10^8 pfu adenovirus was injected into the tail vein. Before and 5 days after the administration of adenovirus expressing active LPL or inactive LPL, mice were fasted for 4 h and a blood sample for the determination of lipids was collected by tail bleeding, using diethyl-*p*-nitrophenyl phosphate (paraoxon; Sigma)-coated heparinized capillary tubes (Hawksley, Sussex, England).

Female *APOE2* mice at 13–18 weeks of age were injected with a dose of 5×10^8 pfu Ad*Apoa5* or AdEmpty. Three hours before this virus injection, the mice were injected with 5×10^8 pfu AdlacZ to saturate the uptake of viral particles by hepatic Kupffer cells (23). Before and 4 days after virus injection, mice were fasted for 4 h and a blood sample for the determination of lipids was collected in paraoxon-coated capillaries by tail bleeding.

Lipid determinations

Plasma was isolated from blood samples obtained from the mice by centrifugation. TG and TC levels were measured enzymatically (Sigma). Human apoE levels were measured by sandwich ELISA as described previously (24). The circulating human apoE level in homozygous *APOE2*-carrying mice was 3.1 ± 0.9 mg/dl.

Lipoproteins were separated using fast-protein liquid chromatography (FPLC). Plasma pools obtained from the various groups of mice were diluted five times using PBS. A volume of 50 μl was injected onto a Superose 6 column (3.2 × 30 mm, AKTA system; Pharmacia, Uppsala, Sweden) to separate lipo-

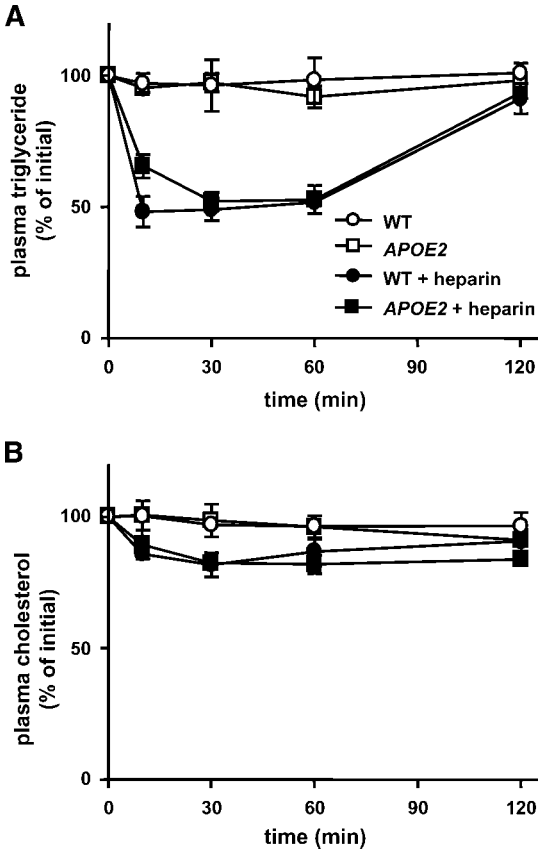

Fig. 1. Plasma lipid levels of apolipoprotein E2 (*APOE2*) mice after heparin treatment. Fasted *APOE2* mice were injected with heparin. Before (time 0) and 10, 30, 60, and 120 min after injection, plasma samples were obtained and assayed for triglyceride (TG; A) and cholesterol (B). The values are represented as percentages of preinjection levels and are means ± SD for n = 4 mice per group. WT, wild type.

protein fractions. Elution fractions of 50 μl were collected and assayed enzymatically for TG and TC levels as described above.

Heparin treatment

Heparin (or vehicle) was administered to *APOE2* mice after a period of 4 h of fasting via intravenous injection of a dose of 0.5 U/g body weight. Blood samples of ~30 μl were drawn via the tail vein at 0, 10, 30, 60, and 120 min after heparin injection using paraoxon-coated capillaries. Plasma TG levels were measured enzymatically as described above.

Fat load

The fat load response was determined in male *APOE2*, *APOE2.Apoc3*$^{+/-}$, and *APOE2.Apoc3*$^{-/-}$ mice aged 13–20 weeks. The mice were fasted overnight and given an intragastric olive oil load (Carbonell, Cordoba, Spain) of 400 μl. Before the olive oil load and 3 h after the load, a blood sample was drawn via the tail vein for plasma TG determination. The circulating TG levels were corrected for the TG level before the fat load.

Statistical analysis

Data were analyzed using the nonparametric Mann-Whitney test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of increased plasma LPL level on lipid levels in *APOE2* mice

Intravenous injection of heparin results in an increase of active LPL in the circulation. Stimulation of LPL activity in *APOE2* mice via injection of heparin (0.5 U/g body weight) reduced the hyperlipidemia in a time-dependent manner (**Fig. 1**). The maximum reduction was observed at 60 min after injection. At this time point, the plasma TG levels decreased by 53% (Fig. 1A) and the TC levels decreased by 18% (Fig. 1B). This effect is similar to the effect of heparin as observed in wild-type mice (Fig. 1).

APOE2 mice were injected with adenovirus expressing enzymatically active and inactive LPL to further investigate the suitability of apoE2-containing particles as substrate for LPL (**Fig. 2**). At day 5 after injection of 5×10^8 pfu Ad*LPL*-inactive, *APOE2* mice exhibited a 129% increase in plasma TG levels (Fig. 2A), whereas TC levels were hardly affected (+12.5%) (Fig. 2B). The increase in plasma TG after Ad*LPL*-inactive administration may be a nonspecific virus-mediated effect but may also result from the opsonization of lipoprotein with inactive protein, thereby

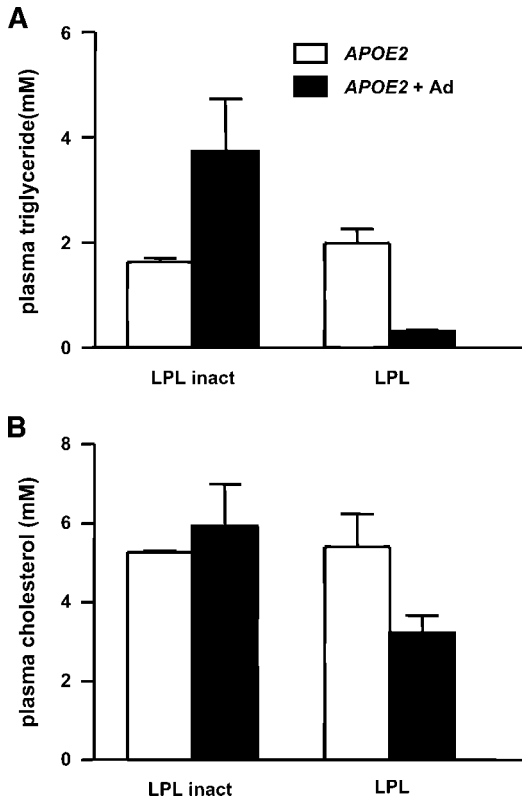

Fig. 2. Plasma lipid levels of *APOE2* mice injected with adenoviral vectors expressing enzymatically active LPL (Ad*LPL*) and inactive LPL (Ad*LPL*-inactive). *APOE2* mice were injected with 5×10^8 plaque-forming units (pfu) Ad*LPL*-inactive or Ad*LPL*. Before (open bars) and at day 5 after adenovirus injection (closed bars), fasted plasma samples were assayed for TG (A) and cholesterol (B). Values are represented as means ± SD for n = 3 mice per group.

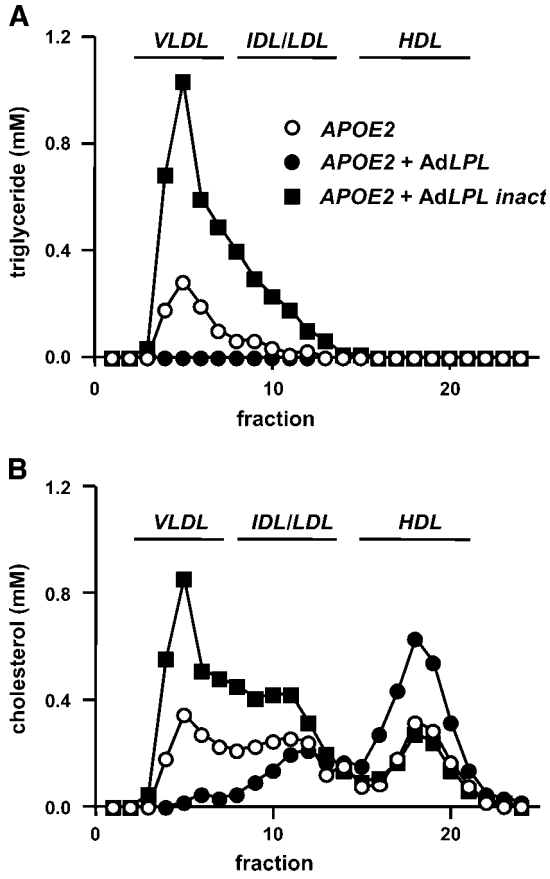

Fig. 3. Lipoprotein profiles of *APOE2* mice injected with Ad*LPL*-inactive and Ad*LPL*. Pooled plasma samples of *APOE2* mice injected with 5×10^8 pfu AdMock, Ad*LPL*-inactive, and Ad*LPL* (n = 3 per group) were subjected to fast-protein liquid chromatography (FPLC), and the elution fractions were assayed for TG (A) and cholesterol (B). IDL, intermediate density lipoprotein.

preventing the interaction with active LPL at the endothelial surface. In contrast, at day 5 after injection of 5×10^8 pfu Ad*LPL*, *APOE2* mice exhibited an 85% decrease in plasma TG levels (Fig. 2A) and a 40% decrease in TC levels (Fig. 2B). The lipoprotein distribution as determined by FPLC showed a decrease in VLDL-TG and VLDL-TC to wild-type levels after injection of Ad*LPL*, indicating an accelerated conversion of apoE2-containing VLDL particles by overexpression of LPL (**Fig. 3**).

Effect of apoC-III deficiency on lipid levels in *APOE2* mice

The main endogenous inhibitor of LPL, apoC-III, was deleted from the genetic background of *APOE2* mice by crossbreeding with *Apoc3*-knockout mice. The effect of *Apoc3* deficiency on *APOE2*-associated hyperlipidemia was investigated in *APOE2* mice heterozygous or homozygous deficient for the endogenous *Apoc3* gene (**Fig. 4**). Surprisingly, the plasma TG levels were not different between *APOE2*, *APOE2.Apoc3*$^{+/-}$, and *APOE2.Apoc3*$^{-/-}$ mice (Fig. 4A). The TC levels in *APOE2* mice showed a tendency toward a decrease by *Apoc3* deficiency (Fig. 4B). No differences in plasma lipid levels were found between male and female mice (data not shown). The distribution of TG and TC over the lipoprotein fractions was measured after separation via FPLC (**Fig. 5**). No differences were observed

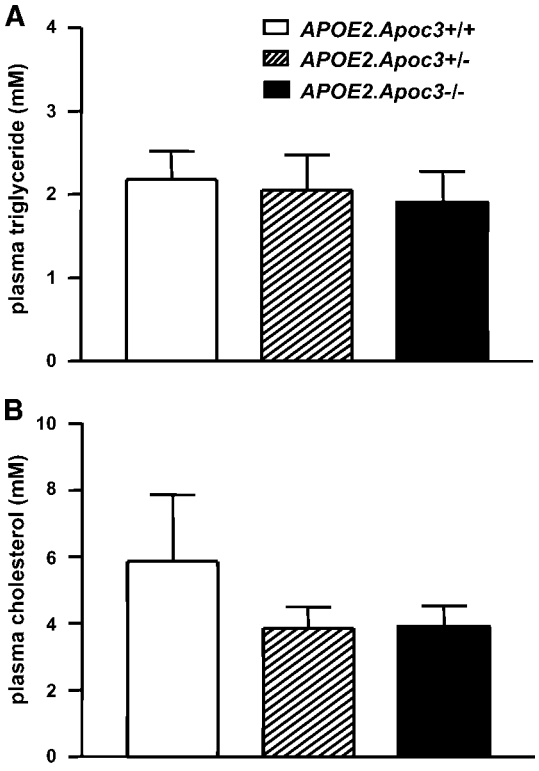

Fig. 4. Plasma lipid levels of *APOE2* mice deficient for *Apoc3*. Fasted plasma samples were obtained from *APOE2* mice (open bars), *APOE2.Apoc3*$^{+/-}$ mice (hatched bars), and *APOE2.Apoc3*$^{-/-}$ mice (closed bars). The samples were assayed for TG (A) and cholesterol (B). Values are represented as means ± SD for n = 5 mice per group.

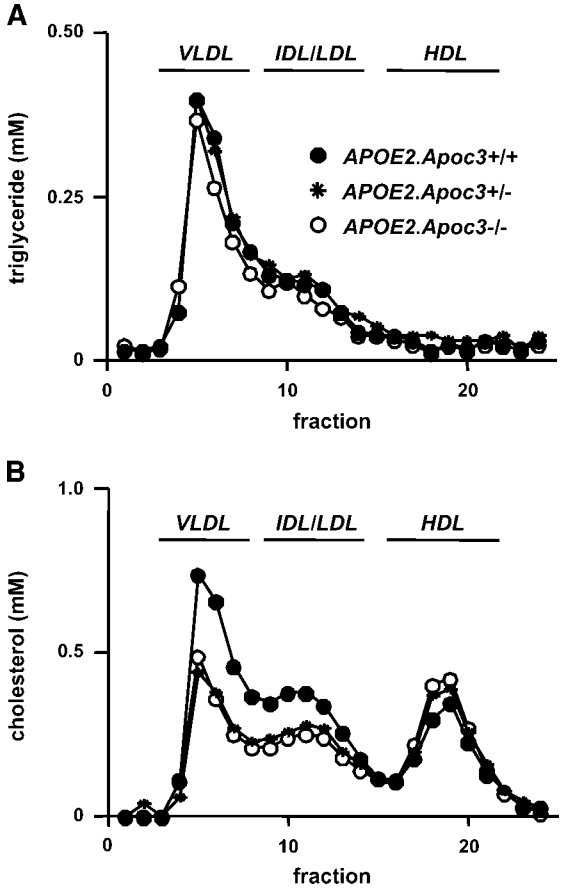

Fig. 5. Lipoprotein profiles of *APOE2* mice deficient for *Apoc3*. Pooled plasma samples of *APOE2*, *APOE2.Apoc3*$^{+/-}$, and *APOE2.Apoc3*$^{-/-}$ mice (n = 5 per group) were subjected to FPLC, and the elution fractions were assayed for TG (A) and cholesterol (B).

between *APOE2*, *APOE2.Apoc3*$^{+/-}$, and *APOE2.Apoc3*$^{-/-}$ mice in TG distribution (Fig. 5A), which is in full accordance with the total plasma TG levels, However, the tendency toward a decrease in TC levels by *Apoc3* deficiency is reflected by a decrease in cholesterol in the VLDL/LDL fractions in the *APOE2.Apoc3*$^{+/-}$ and *APOE2.Apoc3*$^{-/-}$ mice (Fig. 5B).

To further analyze the effect of *Apoc3* deficiency in *APOE2* mice on TG metabolism, mice were given an intragastric olive oil load. The increase in plasma TG levels was measured over a period of 3 h and the TG appearance rate was determined. The increase in TG level over 3 h was not different between *APOE2* mice (0.9 ± 1.6 mM; n = 5), *APOE2.Apoc3*$^{+/-}$ mice (1.1 ± 0.4 mM; n = 5), and *APOE2.Apoc3*$^{-/-}$ mice (1.2 ± 1.5 mM; n = 4).

Effect of adenovirus-mediated expression of *Apoa5* on lipid levels in *APOE2* mice

The activator of LPL, apoA-V, was expressed in *APOE2* mice via a recombinant adenoviral vector (**Fig. 6**). Injection of a moderate dose of Ad*Apoa5* (5×10^8 pfu) reduced plasma TG by 81% ($P < 0.05$) and TC by 41% ($P < 0.05$) compared with AdEmpty. Analysis of lipo-

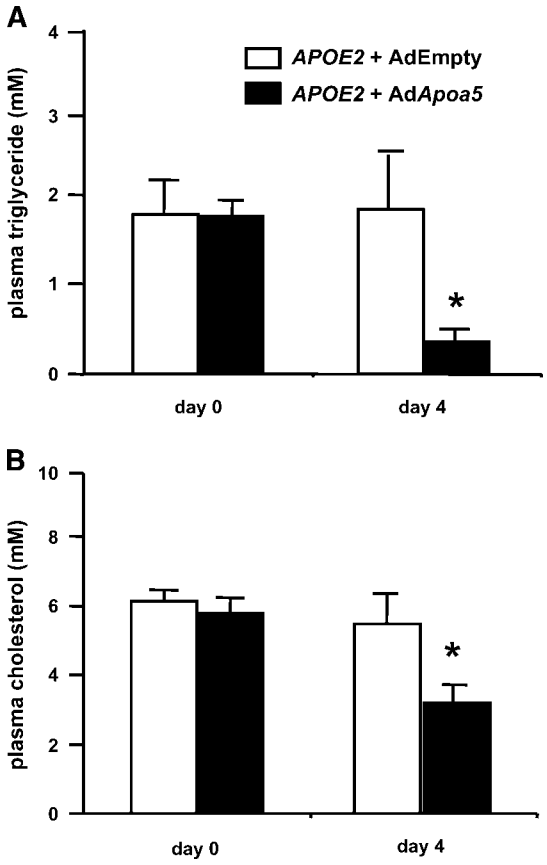

Fig. 6. Plasma lipid levels of *APOE2* mice injected with Ad*Apoa5*. *APOE2* mice were injected consecutively with AdlacZ (5×10^8 pfu) and Ad*Apoa5* or AdEmpty (5×10^8 pfu). Before injection (open bars) and at 4 days after injection (closed bars), fasted plasma was collected from the individual mice and assayed for TG (A) and cholesterol (B). Values are represented as means ± SD for five mice per group. * $P < 0.05$.

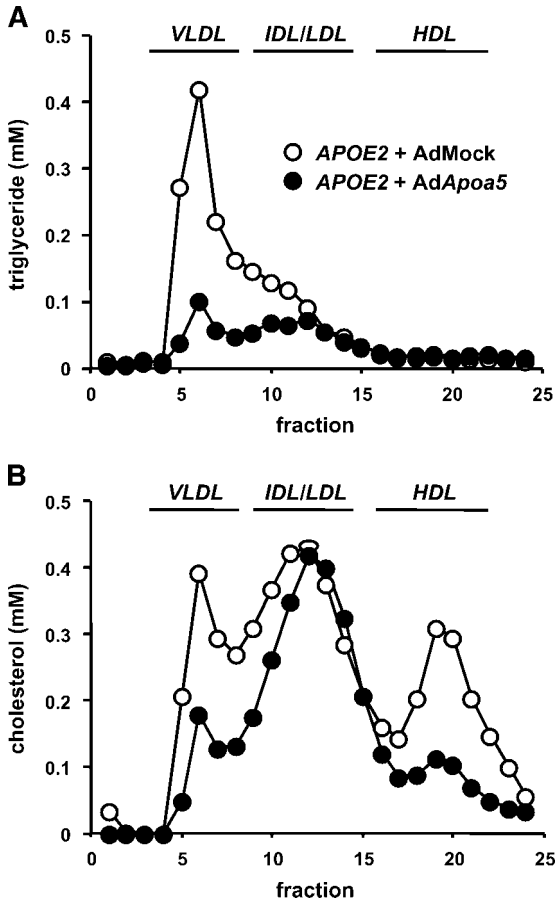

Fig. 7. Lipoprotein profiles of *APOE2* mice injected with Ad*Apoa5*. Pooled plasma samples of *APOE2* mice injected with 5×10^8 pfu AdMock or Ad*Apoa5* (n = 5 per group) were subjected to FPLC, and the elution fractions were assayed for TG (A) and cholesterol (B).

protein fractions separated by FPLC revealed that the apoA-V-mediated reduction of plasma TG was associated with a 4-fold reduction in VLDL-TG, whereas the TG level in the intermediate density lipoprotein/LDL fraction was affected to a minor degree. The reduction in plasma TC level was associated with a 2-fold reduced VLDL-TC level (**Fig. 7**).

DISCUSSION

In the current study, we have addressed the hypothesis that alleviating the apoE2-mediated inhibition of lipolysis can reduce the apoE2-associated hypertriglyceridemia. Using the *APOE2* mouse model, we first increased circulating LPL activity directly via heparin injection. This resulted in a reduction of the TG and TC levels in *APOE2* mice (Fig. 1). Likewise, injection of adenovirus expressing LPL in *APOE2* mice reduced the plasma TG and TC levels (Fig. 2). The reduction in TG and TC was confined mainly to the VLDL-sized fractions (data not shown). These experiments demonstrated that apoE2-containing particles are a suitable substrate for LPL. Subsequently, LPL was stimulated indirectly via its oppositely acting modulators apoC-III and apoA-V. *Apoa5* overexpression did reduce the *APOE2*-associated hypertriglyceridemia in *APOE2*-knockin mice (Fig. 6). In contrast, the *APOE2*-associated hypertriglyceridemia was not affected by *Apoc3* deficiency (Fig. 4). Our data indicate that a direct increase of LPL activity by increasing circulating LPL levels reduces *APOE2*-associated hypertriglyceridemia. The indirect stimulation of LPL activity via *Apoa5* overexpression but not *Apoc3* deficiency ameliorates the *APOE2*-associated hypertriglyceridemia. We conclude that apoA-V is apparently dominant over apoC-III in the improvement of *APOE2*-associated hypertriglyceridemia. Moreover, apoA-V and apoC-III modulate LPL activity via distinct mechanisms.

The effects of Ad*Apoa5* injection on plasma lipid levels of wild-type mice have been reported by us previously (8). At the dose used in the current study (5×10^8 pfu/mouse), Ad*Apoa5* resulted in a significant decrease in TC (68%) and TG (65%) in wild-type mice. In *APOE2* mice, Ad*Apoa5* decreased TG more severely (81%) compared with TC (41%), in agreement with a primary effect of

apoA-V on lipolysis. The effect of apoC-III deficiency on plasma lipid levels of wild-type mice revealed a more dramatic effect on TG levels (74% reduction) compared with TC levels (56% reduction) (15). This is also in agreement with an increased activity of LPL in the absence of apoC-III (11, 12, 15). Compared with wild-type mice, the *APOE2* mice displayed a combined hyperlipidemia, characterized by increased levels of both TC and TG. Because increasing circulating LPL levels decreased the *APOE2*-associated hyperlipidemia, and specifically the hypertriglyceridemia, the identical plasma TG levels of *APOE2*, *APOE2.Apoc3*$^{+/-}$, and *APOE2.Apoc3*$^{-/-}$ mice are a strong indication that apoC-III deficiency truly has very little effect on the *APOE2*-associated hypertriglyceridemia. Interestingly, the *APOE2*-associated hypercholesterolemia seems reduced by *Apoc3* deficiency (Fig. 4). It has been reported that, besides inhibiting LPL, apoC-III can also directly inhibit the hepatic uptake of apoE-containing lipoproteins by the liver (25). Apparently, *Apoc3* deficiency in *APOE2* mice does not affect LPL activity, as is evident from unaltered plasma TG levels, but may enhance the removal of cholesterol-enriched lipoprotein remnant particles, as is evident from reduced (V)LDL-cholesterol levels (Fig. 5).

Addition of apoE to lipoproteins results in a decrease in the LPL-mediated TG hydrolysis (26–28). This can at least partially explain the hypertriglyceridemia that is found in *APOE2*-associated familial dyslipidemia, which is characterized by plasma accumulation of apoE-enriched lipoproteins. It has been proposed that the inhibition of LPL activity is caused by displacement of the LPL coactivator apoC-II from the apoE2-rich lipoprotein particles (5). However, this is difficult to reconcile with the observation that indirect stimulation of LPL activity via apoA-V overexpression ameliorates the *APOE2*-associated hyperlipidemia, especially because it has been demonstrated that the LPL-activating effect of apoA-V is dependent on the presence of apoC-II (8). Thus, other mechanisms might underlie the inhibitory effect of apoE2 on LPL activity.

Under normal conditions, LPL-mediated TG hydrolysis takes place mainly at the endothelial cell surface and thus may be affected by the interaction between the TG-containing particle and the cell surface where LPL is localized. This interaction involves the association of TG-rich particles and endothelial surface-bound heparan sulfate proteoglycans (HSPGs) via apoE (29). It has been shown that apoE2 is partly defective in the association with HSPGs (30), and this could also explain part of the apoE2-associated hypertriglyceridemia. In agreement with this hypothesis, it has been found in vitro that VLDL obtained from *APOE2* homozygous familial dyslipidemia patients is effectively lipolyzed by LPL in solution but is poorly lipolyzed by HSPG-bound LPL (6). Thus, apoE2-containing VLDL may be defective in the physical association with the endothelial surfaces where LPL-mediated TG hydrolysis takes place in vivo. This would explain why both additional soluble LPL via adenovirus-mediated gene transfer and increasing soluble LPL via heparin injection rescue the apoE2-associated hyperlipidemia. Intriguingly, this explanation is also in agreement with the observation that additional apoA-V rescues the apoE2-associated hyperlipidemia. It was found recently that the LPL-activating effect of apoA-V involves enhanced binding to HSPGs (10, 31). Thus, additional apoA-V on the TG-rich particle apparently overcomes the apoE2-mediated inhibition of HSPG binding. It is interesting that apoC-III deficiency cannot overcome this binding defect, despite the postulated inhibition of HSPG binding by apoC-III (14, 32). However, the in vivo contribution of HSPGs to the lipolysis of TG-rich lipoprotein particles still remains to be determined.

Apart from a stimulatory effect on LPL, it may be postulated that the decrease in plasma TG of *APOE2* mice after the expression of apoA-V results from a decrease in the VLDL-TG secretion rate by the liver. We showed previously a 30% decreased VLDL-TG secretion rate after adenovirus-mediated overexpression of *Apoa5* in wild-type C57BL/6 mice (8), whereas others have found no effects of apoA-V on VLDL production in either *APOA5* transgenic mice (33) or *Apoa5*$^{-/-}$ mice (17). Intriguingly, in the *APOE2* mice, we observed no differences in the VLDL-TG secretion rate after injection of Ad*Apoa5* or AdEmpty (data not shown). At present, we have no explanation for these apparent discrepancies but cannot exclude the possibility that apoA-V has additional unrecognized functions.

Extrapolating our mouse model data to the human, it seems likely that variation in apoA-V level will have a pronounced effect on the expression of hypertriglyceridemia. Surprisingly, we and others have found that in humans, plasma TG and apoA-V levels are positively correlated (34–36). This would appear to be a clear discrepancy between mouse and human. However, it should be noted that the human data are derived from correlation studies, whereas the mouse data are derived from direct interventions in plasma apoA-V levels. Without a complete understanding of the role and metabolism of apoA-V, the apparent discrepancy between circulating levels of apoA-V and hypertriglyceridemia remain to be resolved.

Polymorphisms in both the *APOA5* and *APOC3* genes have been associated with hypertriglyceridemia (19, 37–41). Because both genes are expressed in the same gene cluster and have opposing effects on TG levels, it has been hypothesized that *APOA5* and *APOC3* act synergistically (7, 33). However, our current data clearly indicate that apoA-V and apoC-III affect different steps in the conversion of TG-rich lipoproteins to remnants.

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